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(54) Title: ENHANCED PRODUCTION OF TAXANES BY CELL CULTURES OF *TAXUS* SPECIES

(57) Abstract

This invention provides methods whereby taxol, baccatin III, and other taxol-like compounds, or taxanes, can be produced in very high yield from all known *Taxus* species, e.g., *brevifolia*, *canadensis*, *cuspidata*, *baccata*, *globosa*, *floridana*, *wallichiana*, *media* and *chinensis*. Particular modifications of culture conditions (i.e., media composition and operating modes) have been discovered to enhance the yield of various taxanes from cell culture of all species of *Taxus*. Particularly preferred enhancement agents include silver ion or complex, jasmonic acid (especially the methyl ester), auxin-related growth regulators, and inhibitors of the phenylpropanoid pathway, such as 3,4-methylenedioxy-6-nitrocinnamic acid. These enhancement agents may be used alone or in combination with one another or other yield-enhancing conditions. While the yield of taxanes from plant cell culture of *T. chinensis* is particularly enhanced by use of one or more of these conditions, yield of taxanes for all *Taxus* species has been found to benefit from use of these conditions.

CLAIMS:

1. A method for producing taxanes in high yields in cell culture of a *Taxus* species comprising: cultivating in suspension culture, in one or more nutrient media under growth and product formation conditions, cells of a *Taxus* species derived from callus or suspension cultures, and recovering one or more taxanes from said cells or said medium of said cell culture, or both,

wherein the one or more nutrient media contain an inhibitor of phenylpropanoid metabolism.

2. The method of claim 1, wherein the inhibitor of phenylpropanoid metabolism is selected from 3,4-methylenedioxy-6-nitrocinnamic acid, 3,4-methylenedioxycinnamic acid, , 3-[3,4-methylenedioxyphenyl] propionic acid, 3,4-methylenedioxyphenylacetic acid, 4-Fluoro-L-Phenylalanine, 4-Hydroxyphenylpyruvic acid, 4-Fluoro-DL-Tyrosine, Trans 3,4-Dimethoxycinnamic acid, phenylpropiolic acid, L-2-Hydroxy-3-Phenylpropionic acid, 2-hydroxy-4,6-dimethoxybenzoic acid, SKF-525A, vinylimidazole, ammonium oxalate, sinapic acid, and 1-aminobenzotriazole.

3. The method according to claim 1, wherein at least one of the one or more nutrient media comprises an enhancement agent selected from:

a) an inhibitor of ethylene action;

b) jasmonate-related compound; and

c) an auxin-related growth regulator.

4. The method of claim 1, wherein the one or more nutrient media contain a silver-containing compound, or a silver complex, or a silver ion.

5. The method of claim 3, wherein at least one of the one or more nutrient media contain jasmonic acid or an alkyl ester thereof.

6. The method of claim 5, wherein the alkyl group esterified to jasmonic acid has from one to six carbon atoms.

7. The method of claim 5, wherein the one or more nutrient media further contain a silver-containing compound, a silver complex or silver ion.

8. The method according to claim 3, wherein the auxin-related growth regulator is 1-Naphthaleneacetic acid, 2-Naphthaleneacetic acid, 1-Naphthaleneacetamide / Naphthylacetamide, N-(1-Naphthyl)phthalamic acid, 1-Naphthoxyacetic acid, 2-Naphthoxyacetic acid, beta-Naphthoxyacetic acid, 1-Naphthoxyacetamide, 3-Chlorophenoxyacetic acid, 4-Chlorophenoxyacetic acid, 3-Iodophenoxyacetic acid, Indoleacetamide, Indoleacetic acid, Indoylacetate, Indoleacetyl leucine, Gamma-(3-Indole)butyric acid, 4-Amino-3,5,6-trichloropicolinic acid, 4-Amino-3,5,6-trichloropicolinic acid methyl ester, 3,6-Dichloro-o-anisic acid, 3,7-Dichloro-8-quinolinecarboxylic acid, Phenylacetic acid, 2-Iodophenylacetic acid, 3-Iodophenylacetic acid, 2-Methoxyphenylacetic acid, Chlorpropham, 4-chloroindole-3-acetic acid, 5-Chloroindole-3-acetic acid, 3-chloroindole-3-acetic acid, 5-Bromo-4-chloro-3-indoyl butyrate, Indoleacetyl phenylalanine, Indoleacetyl glycine, Indoleacetyl alanine, 4-chloroindole, p-chlorophenoxyisobutyric acid, 1-pyrenoxylbenzoic acid, Lysophosphatidic acid, 1-naphthyl-N-methylcarbamate, and Ethyl-5-chloro-1H-Indazole-3-ylacetate-3-Indolebutanoic acid. 9. A method for producing taxanes in high yields in cell culture of a *Taxus* species comprising: cultivating in suspension culture, in one or more nutrient media under growth and product formation conditions, cells of a *Taxus* species derived from callus or suspension cultures, and recovering one or more taxanes from said cells or said medium of said cell culture, or both,

wherein the one or more nutrient media contain silver at a concentration of 900μM or less in the form of a silver-containing compound, or a silver complex, or a silver ion, and at least one of the one or more nutrient media comprises an enhancement agent selected from:

a) jasmonic acid or an ester of jasmonic acid; and

b) an auxin-related growth regulator.

10. The method according to claim 9, wherein the enhancement agent is jasmonic acid or an ester of jasmonic acid, and the molar ratio of silver to enhancement agent is less than 9.5.

11. The method according to claim 9, wherein the enhancement agent is an auxin-related growth regulator, and the molar ratio of enhancement agent to silver is at least 0.011.

12. The method according to claim 1 or claim 9, wherein the one or more nutrient media also include a taxane precursor.

13. The method of claim 12, wherein the taxane precursor is α-phenylalanine, β-phenylalanine, or a mixture thereof.

15. The method according to claim 1 or claim 9, wherein the one or more nutrient medium also contains glutamine.

16. The method according to claim 1 or claim 9, wherein the one or more nutrient medium also contains glutamic acid, aspartic acid, or a mixture thereof.

17. The method according to claim 1 or claim 9, wherein the one or more nutrient media includes maltose as a carbon source.

18. The method according to claim 1 or claim 9, wherein the one or more nutrient media includes sucrose as a carbon source.

19. The method according to claim 1 or claim 9, wherein the one or more nutrient media includes glucose, fructose, or a mixture thereof, as a carbon source.

20. The method according to claim 16, wherein maltose, sucrose, glucose, fructose, or a mixture thereof, is the primary carbon source.

21. The method according to claim 1 or claim 9, wherein the nutrient medium is the same for cell culture growth and for taxol and taxane production.

22. The method of claim 1 or claim 9, wherein production of said one or more taxanes is induced by changing composition of the nutrient medium.

23. The method according to claim 22, further comprising exchanging nutrient medium at least once during taxane production.

24. The method according to claim 1 or claim 9, further comprising exchanging nutrient medium at least once during the cultivating step.

25. The method according to claim 1 or claim 9, further comprising removal of taxane from the culture during taxane production.

26. The method of claim 1 or claim 9, wherein cells of said *Taxus* species are cultivated by a fed-batch process.

27. The method according to claim 1 or claim 9, having a product formation period, wherein the volumetric productivity of taxanes is at least 15 mg/L/day averaged over the period of product formation.

28. The method according to claim 1 or claim 9, wherein taxol is recovered from said cells or said medium of said cell culture, or both.

29. The method according to claim 28, wherein the volumetric productivity of taxol is at least 10 mg/L/day computed for the period of taxol production.

30. The method according to claim 1 or claim 9, wherein baccatin III is recovered from said cells or said medium of said cell culture, or both.

31. The method according to claim 30, wherein the volumetric productivity of baccatin III is at least 15 mg/L/day computed for the period of taxane production.

32. The method according to claim 1 or claim 9, wherein the *Taxus* species is *T. brevifolia, T. canadensis, T. chinensis, T. cuspidata, T. baccata, T. globosa, T. floridana, T. wallichiana,* or *T. media*.

33. The method according to claim 1 or claim 9, wherein the cells of a *Taxus* species produce taxol above background by ELISA in callus culture or suspension culture in medium that contains no enhancement agents.

34. The method according to claim 1 or claim 9, wherein the cells of a *Taxus* species produce taxanes in suspension culture at an average volumetric productivity of 10mg/L/day in a medium containing silver thiosulfate, methyl jasmonate and 1-naphthaleneacetic acid.

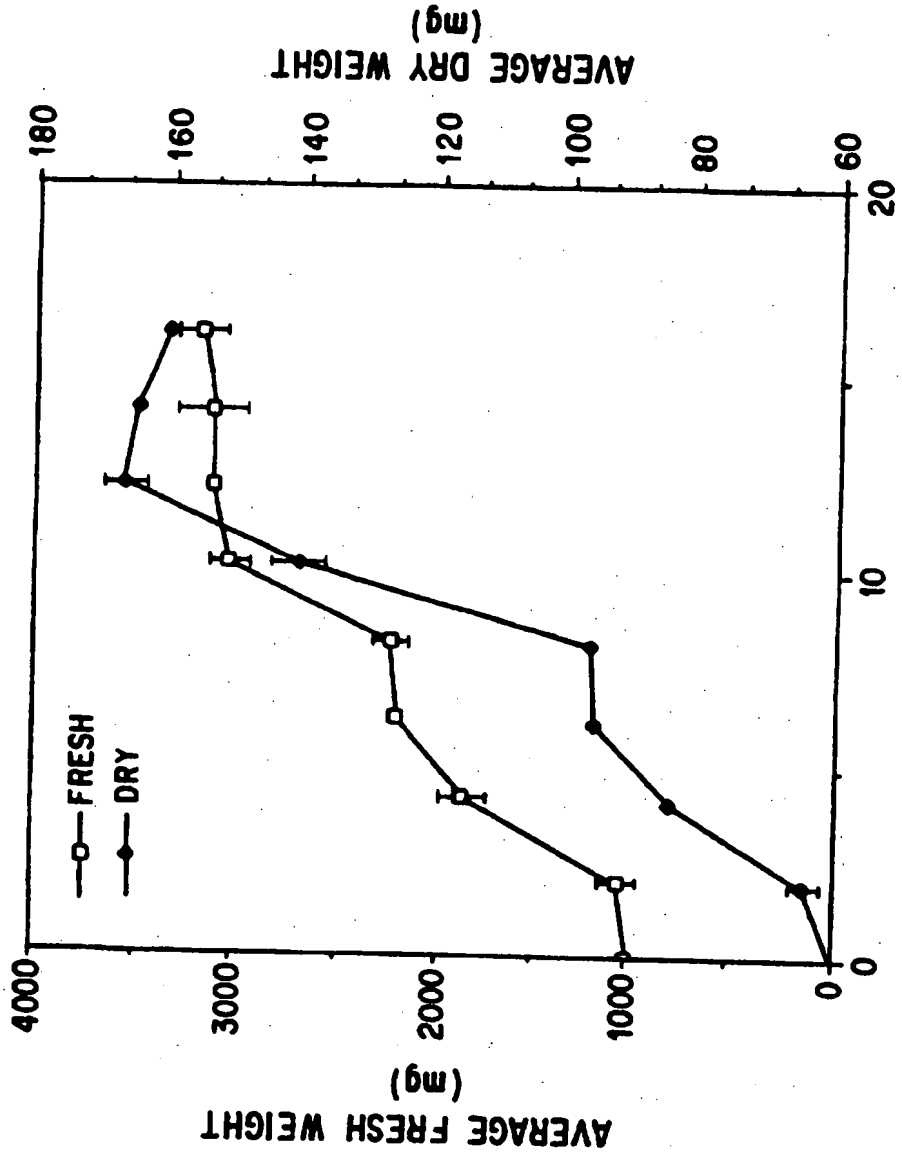
AVERAGE DRY WEIGHT
(mg)
180
160
140
120
100
80
60
20
10
0
DAYS
FRESH
DRY
4000
3000
2000
1000
0
(mg)
AVERAGE FRESH WEIGHT

FIG. 1

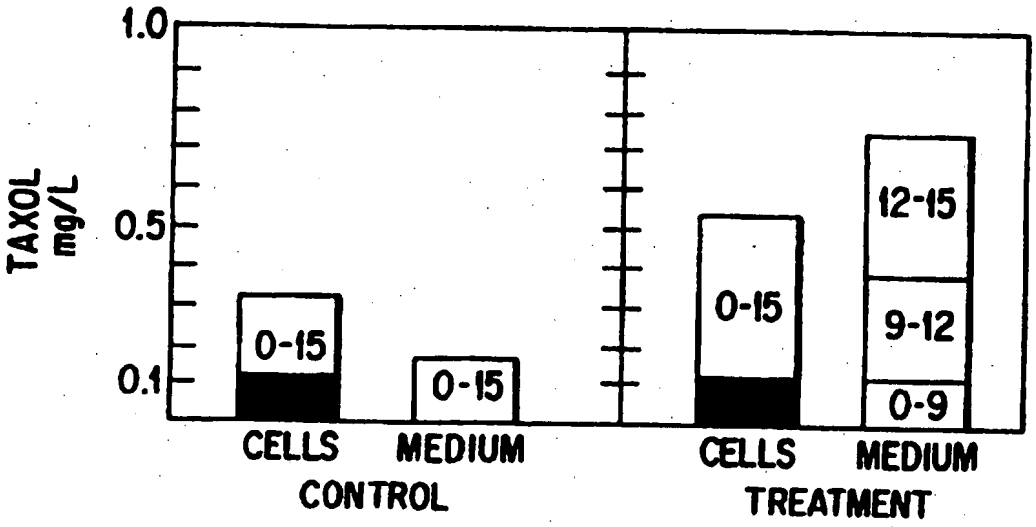
1.0
0.5
0.1
TAXOL mg/L
0-15
0-15
0-15
12-15
9-12
0-9
CELLS
MEDIUM
CONTROL
CELLS
MEDIUM
TREATMENT

FIG. 2A

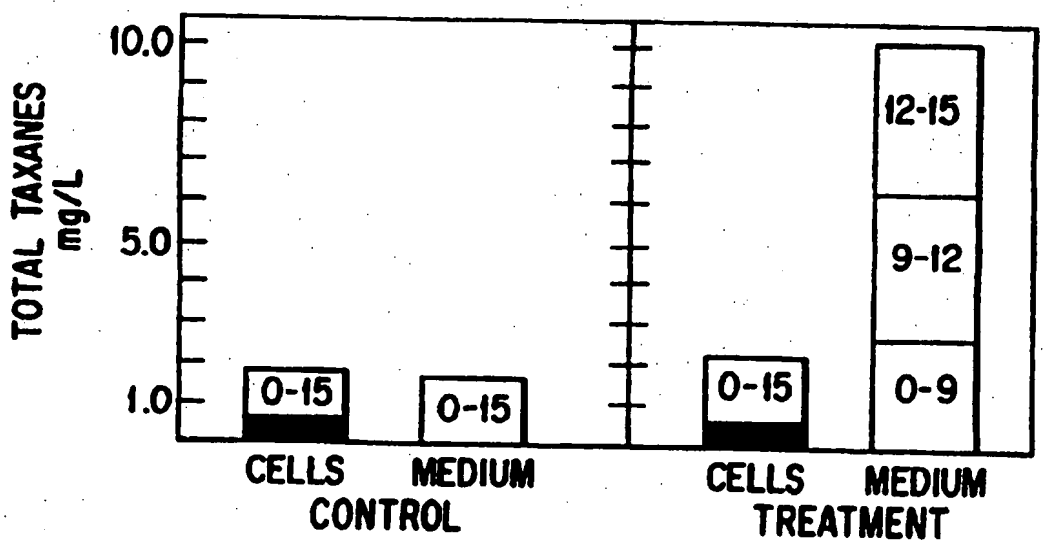
10.0
5.0
1.0
TOTAL TAXANES mg/L
0-15
0-15
0-15
12-15
9-12
0-9
CELLS
MEDIUM
CONTROL
CELLS
MEDIUM
TREATMENT

FIG. 2B

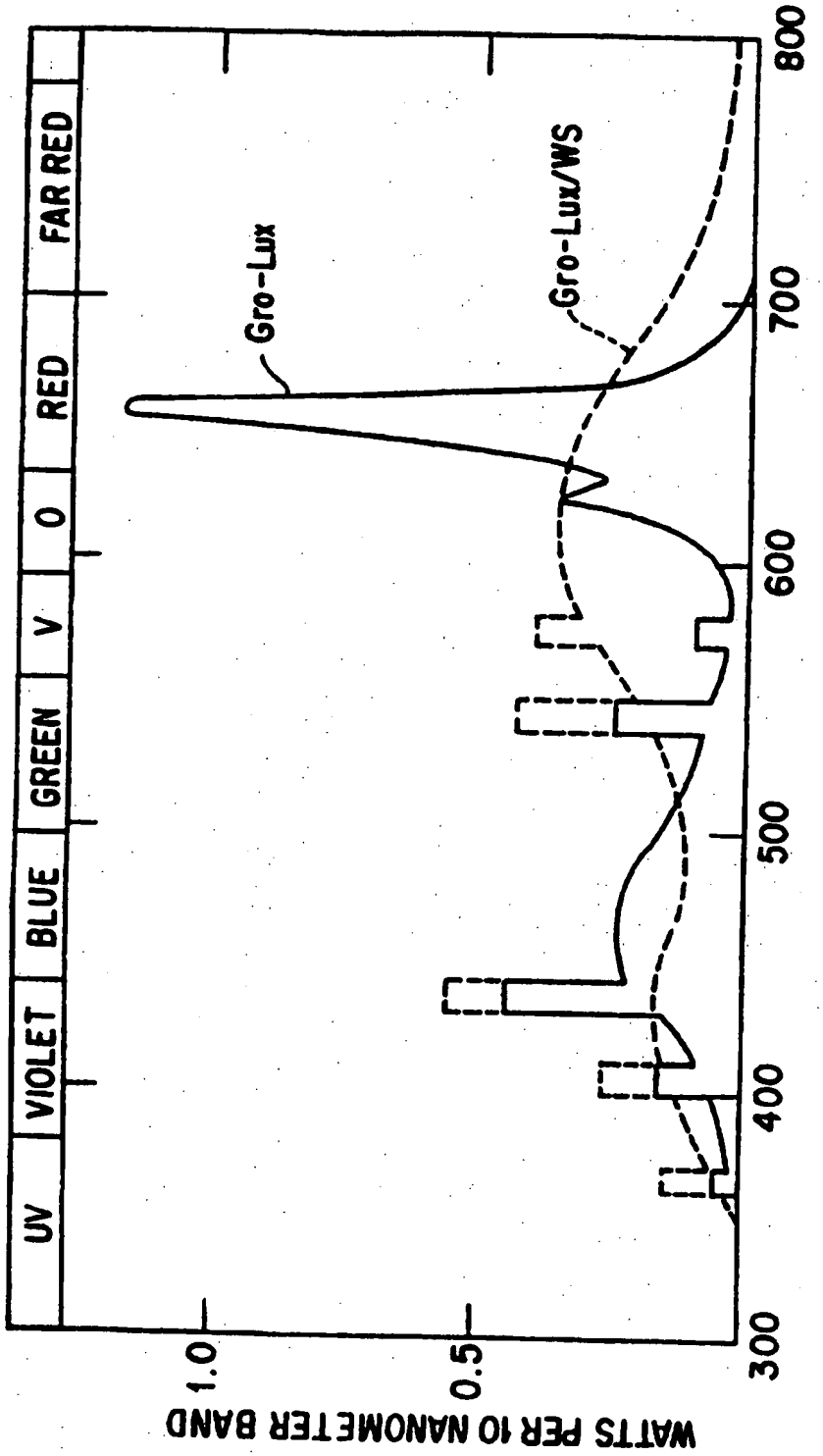
UV
VIOLET
BLUE
GREEN
V
O
RED
FAR RED
Gro-Lux
Gro-Lux/WS
300
400
500
600
700
800
0.5
1.0
WATTS PER 10 NANOMETER BAND

FIG. 3

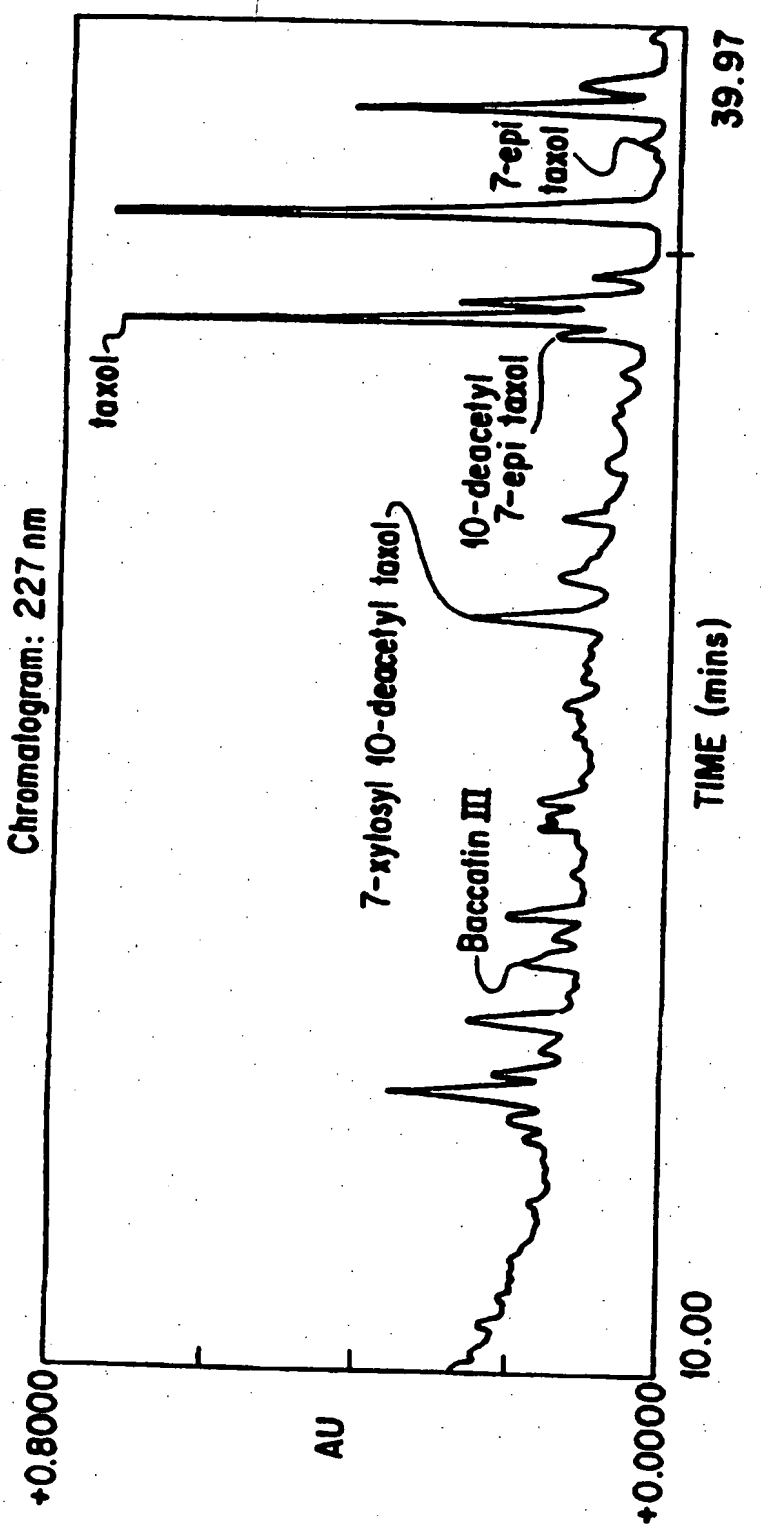
Chromatogram: 227 nm
+0.8000
AU
+0.0000
10.00
TIME (mins)
39.97
taxol
7-xylosyl 10-deacetyl taxol
Baccatin III
10-deacetyl 7-epi taxol
7-epi taxol

FIG. 4A

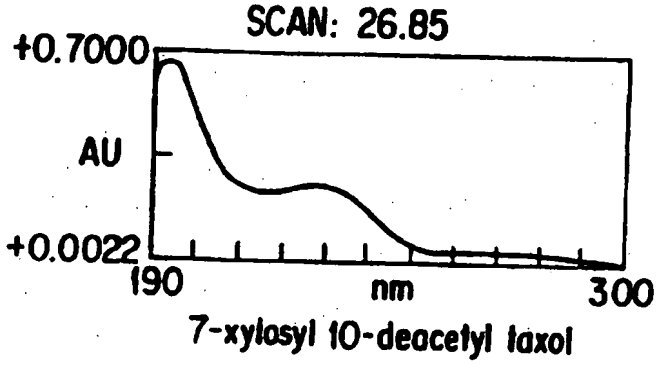

FIG. 4B

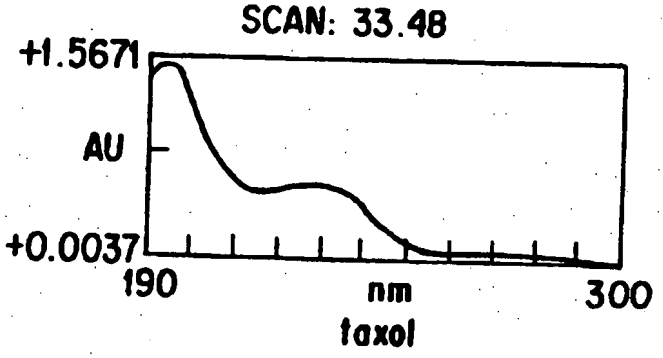

FIG. 4C

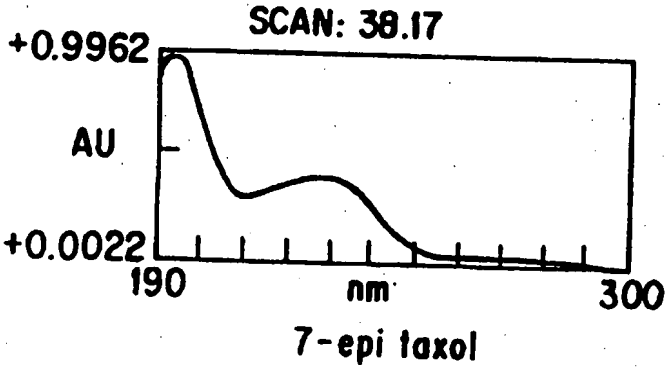

FIG. 4D

		DAY 25			DAY 42	
COMPOUND	% D. W.	mg/L	% Extracellular	% D. W.	mg/L	% Extracellular
10-Deacetylbaccatin III	0.0000	0.00		0.0000	0.00	
Baccatin III	0.0184	10.43	10.57	0.0420	19.83	14.72
7-Xylosyl-10-deacetyltaxol	0.0127	7.19	24.62	0.0283	13.38	45.81
10-deacetyltaxol	0.0122	6.95	17.37	0.0127	5.99	0.00
Cephalomannine	0.0000	0.00		0.0119	5.60	86.02
10-deacetyl-7-epitaxol	0.0081	4.61	62.42	0.0275	12.99	72.59
Taxol	0.0427	24.25	70.95	0.3244	153.34	87.52
7-Epitaxol	0.0122	6.92	84.61	0.0154	7.26	85.28
TOTAL-Unknown	0.0452	25.67		0.1625	76.83	
TOTAL Taxanes	0.1515	86.84		0.6245	295.23	

FIG. 5A

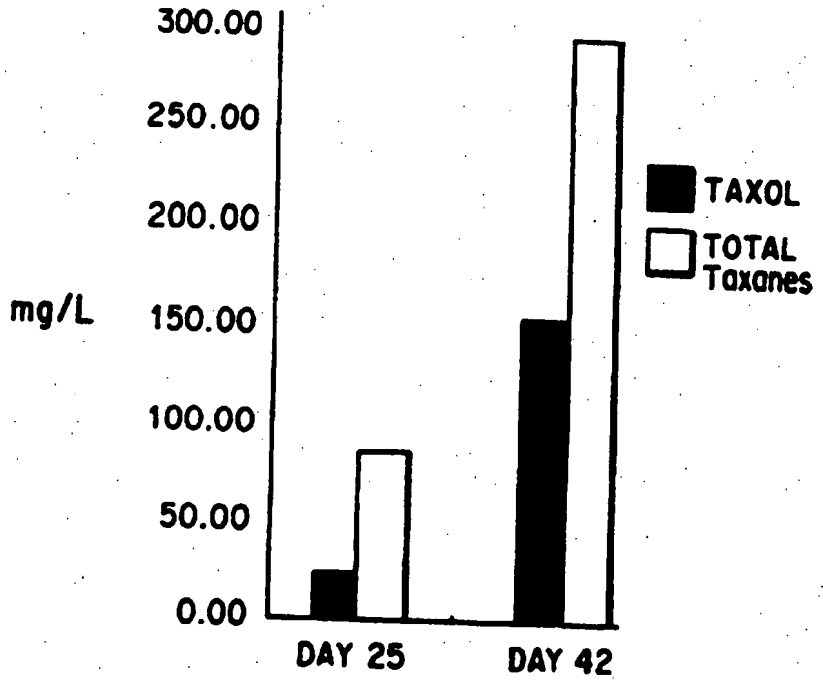
300.00
250.00
200.00
mg/L
150.00
100.00
50.00
0.00
DAY 25
DAY 42
TAXOL
TOTAL
Taxanes

FIG. 5B

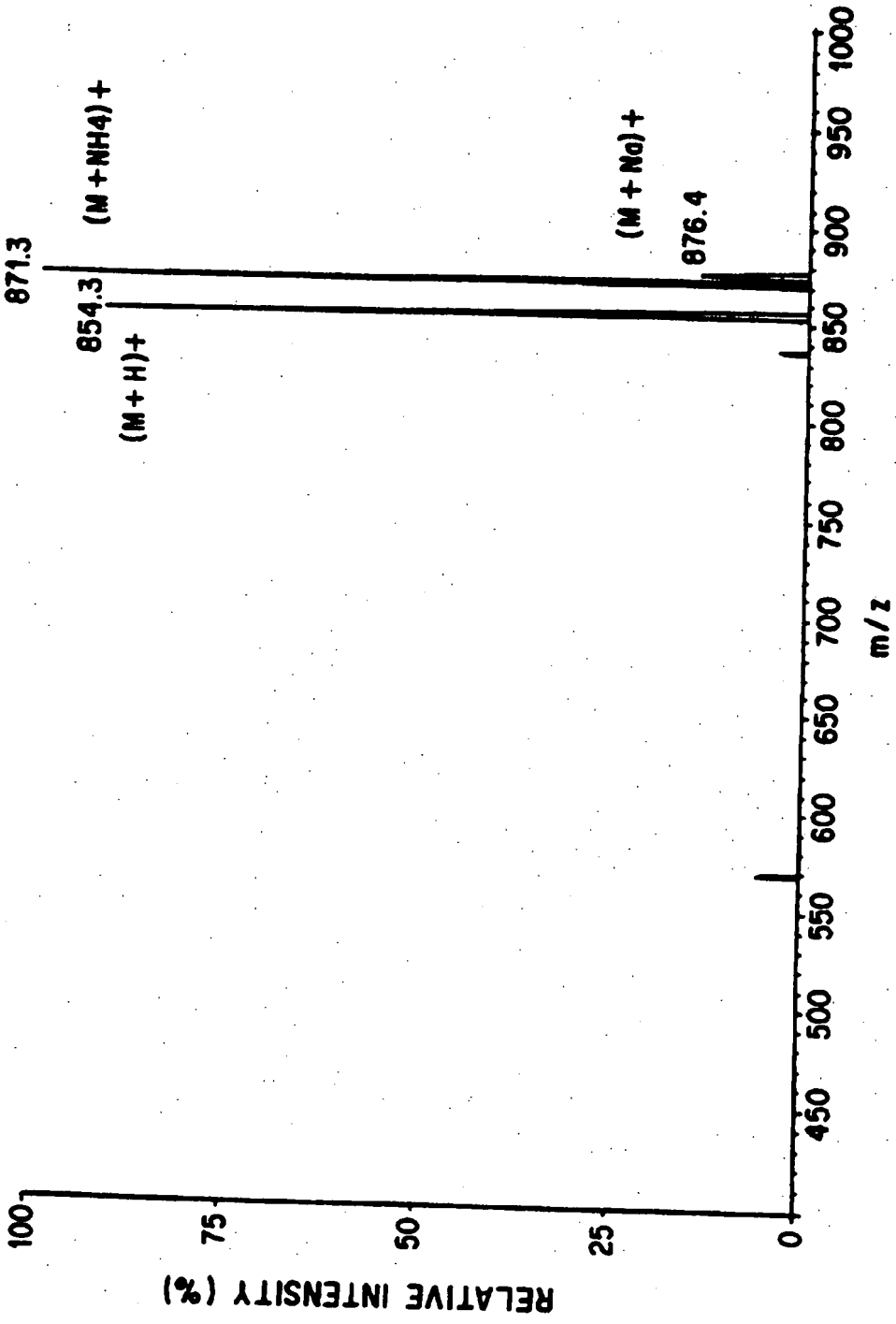
871.3
(M+NH4)+
854.3
(M+H)+
876.4
(M+Na)+
100
75
50
25
0
RELATIVE INTENSITY (%)
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

FIG. 6A

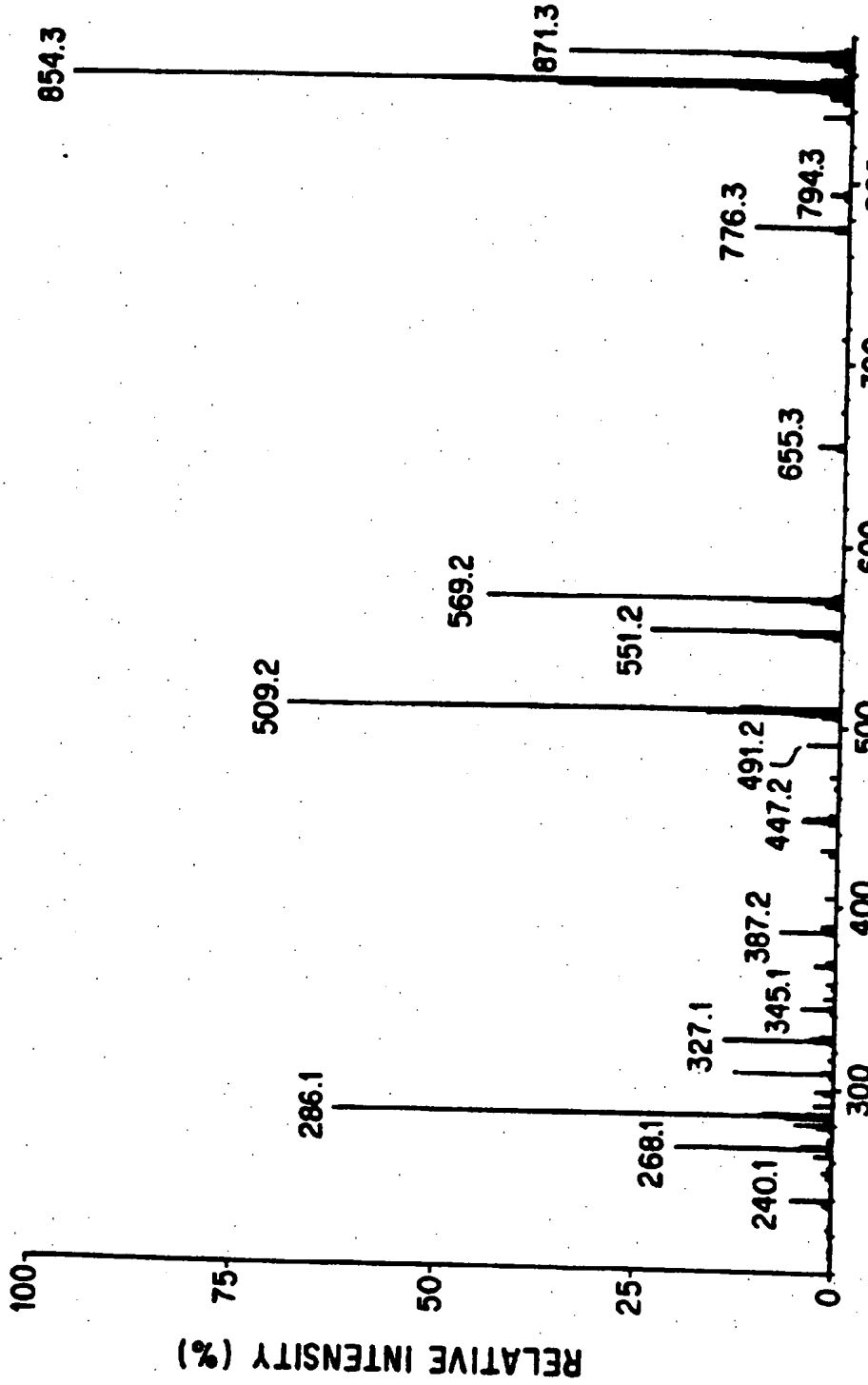
RELATIVE INTENSITY (%)
100
75
50
25
0
m/z
300
400
500
600
700
800
854.3
871.3
794.3
776.3
655.3
569.2
551.2
509.2
491.2
447.2
387.2
345.1
327.1
286.1
268.1
240.1

FIG. 6B

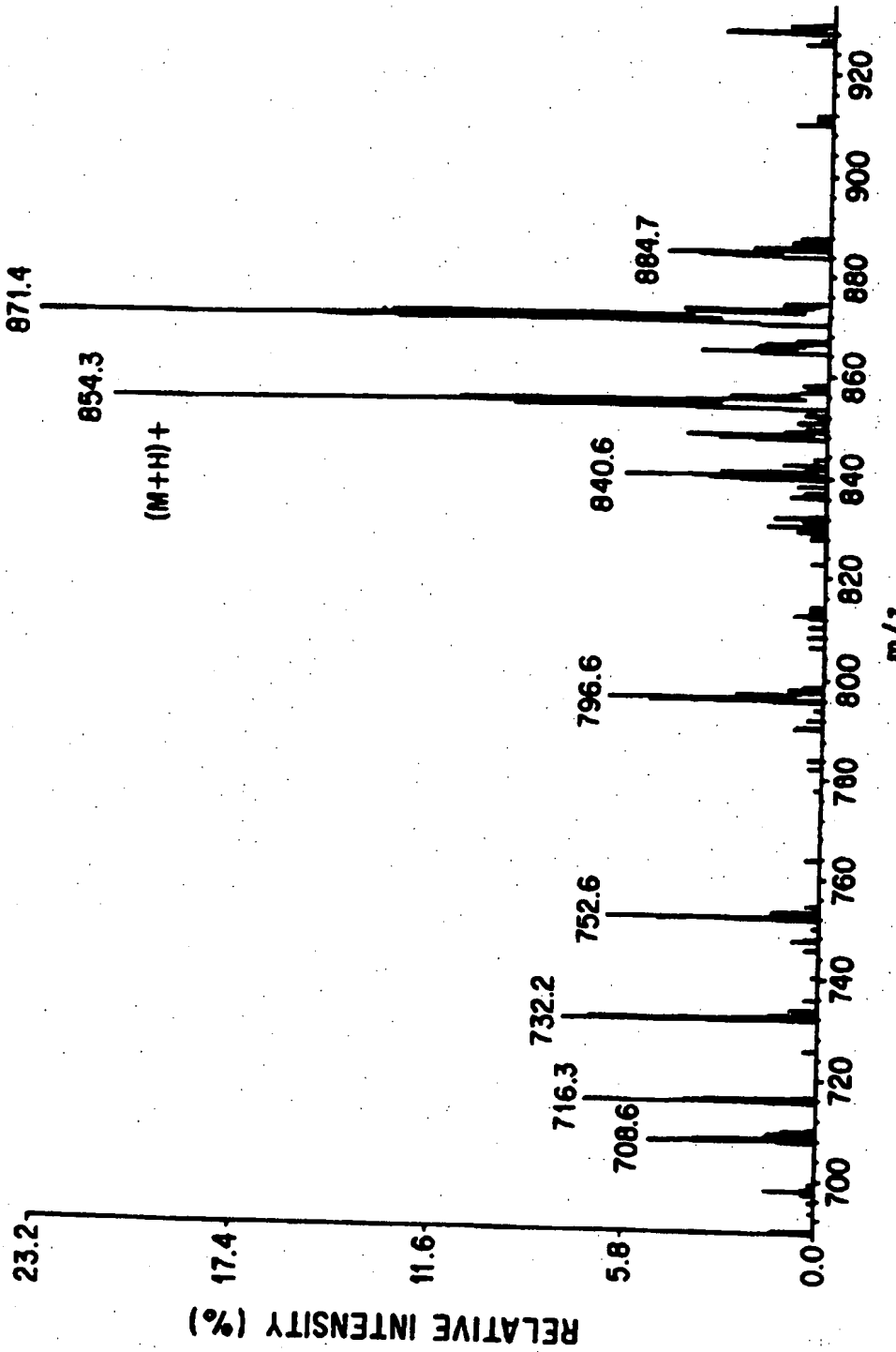
RELATIVE INTENSITY (%)
23.2
17.4
11.6
5.8
0.0
700
720
740
760
780
800
820
840
860
880
900
920
m/z
871.4
854.3
(M+H)+
884.7
840.6
796.6
752.6
732.2
716.3
708.6

FIG. 6C